833 ml. of hydrogen being absorbed in 100 minutes. The solution was filtered and concentrated leading to diethyl α-acetamidomethylmalonate (6.75 g., 0.028 mole), 93% yield, m.p. 38–38.5° from hexane–ether.

Anal. Calcd. for $C_{10}H_{17}NO_5$: C, 51.94; H, 7.36; N, 6.06. Found: C, 51.94; H, 7.43; N, 6.22.

Dipotassium α-Acetamidomethylmalonate.—A solution of 1.15 g. (0.005 mole) of diethyl α-acetamidomethylmalonate in 2 ml. of ethanol was treated with 22.6 ml. of 0.464 *N* potassium hydroxide (0.0104 mole) in ethanol. A precipitate was collected after 4 hr. and washed with ethanol; 1.20 g. (0.0043 mole), 86% yield, dec. 285°.

Anal. Calcd. for $C_6H_7NO_5K_2 \cdot 2H_2O$: C, 25.08; H, 3.48; N, 4.88. Found: C, 24.38; H, 3.65; N, 5.32.

Ethyl Hydrogen α-Acetamidomethylmalonate.—A solution of 4.62 g. (0.020 mole) of diethyl α-acetamidomethylmalonate in 80 ml. of ethanol was treated gradually with stirring over a period of 3 hr. with 41.0 ml. of 0.464 *N* potassium hydroxide (0.019 mole) in ethanol. The solution was evaporated to dryness at room temperature, and the residue was washed with ether. The residue was dissolved in a little water, 19.0 ml. of 1 *N* hydrochloric acid was added, the solution was concentrated to dryness at 1 mm., and the residue was extracted with acetone. The extract was concentrated to dryness, and the oily residue was treated with hexane, slowly crystallizing, giving the monoester (3.75 g., 0.0185 mole), 92% yield, melting 65–75° dec. This was converted to a derivative:

1-(Ethyl α-acetamidomethylmalonyl)-1,3-bis-(*p*-dimethylaminophenyl)-urea.—Ethyl hydrogen α-acetamidomethylmalonate (1.0 g., 4.9 mmoles) in 10 ml. of acetone was treated with 1.2 g. (4.3 mmoles) of 1,3-bis-(dimethylaminophenyl)-carbodiimide[16] in 20 ml. of acetone for 20 hr. atr oom temperature. The solution was decanted from a precipitate and evaporated to form a solid residue (1.64 g., 3.4 mmoles), 80% yield, m.p. 153–154° dec. from benzene. The infrared spectrum was determined in chloroform: 2.89(w), 3.06(w), 3.35(m), 5.78(s), 5.94(s), 6.17(m), 6.57(s), 6.90(w), 7.38-(m), 7.57(w), 8.13(m), 10.60(m) μ.

Anal. Calcd. for $C_{25}H_{33}N_5O_6$: C, 62.11; H, 6.83; N, 14.49. Found: C, 61.65; H, 6.85; N, 14.00.

Hydrolysis of Diethyl α-Acetamidomethylmalonate by α-Chymotrypsin.—(1) A solution of 0.1159 g. (0.500 mmole) of the ester in 15 ml. of water and 2 ml. of 0.1 *M* Na_2HPO_4 buffer was placed in the *p*H stat at *p*H 8.1 and found not to hydrolyze. Enzyme was added, 0.1036 g., and 0.2 *N* sodium hydroxide was added automatically, 2.17 ml. (0.434 mmole) being consumed in 22 hr., 87% reaction. The polarimeter reading was the same as that of the α-chymotrypsin alone.

(2) A solution of 0.922 g. (4.00 mmoles) of the ester and 0.200 g. of the enzyme in 20 ml. of water and 0.5 ml. of buffer was allowed to react in the *p*H stat, 3.076 ml. of 1.0 *N* sodium hydroxide being consumed in 21 hr., 77% reaction, α_{obsd} −0.88°, possible contribution due to hydrolysis products +0.23°. The solution was taken to dryness in vacuum, and the residue was washed with ether to remove unreacted ester. The residue was dissolved in water, treated with 3.0 ml. of 1.0 *N* hydrochloric acid and taken to dryness again. The residue was extracted with acetone, which was concentrated, leading to a residue which was crystallized from chloroform–hexane; ethyl hydrogen α-acetamidomethylmalonate, 0.617 g. (3.04 mmoles), 99% yield, m.p. 70–80° dec. This was dissolved in chloroform, treated with Norit A, filtered, diluted to 10 ml. and examined in the polarimeter, α_{obsd} +0.03°. The chloroform was evaporated, and the residue was triturated with hexane and dried, 0.587 g. being recovered. A portion of this (0.503 g., 2.48 mmoles) was dissolved in 3 ml. of water, brought to *p*H 7 with 2.27 ml. of 1.0 *N* sodium hydroxide, filtered and examined in the polarimeter, α_{obsd} −0.06°. The solution was reacidified with 2.1 ml. of 1.0 *N* hydrochloric acid, taken to dryness and extracted with acetone leading to recovery of 0.484 g. (2.38 mmoles) of the monoester. This was dissolved in 4 ml. of acetone and treated with 0.586 g. (2.10 mmoles) of 1,3-bis-(*p*-dimethylaminophenyl)-carbodiimide in 10 ml. of acetone for 20 hr. The solution was filtered and concentrated, leading to the ureide (0.790 g., 1.64 mmoles), 78% yield, crystallized from benzene, m.p. and mixed m.p. with an authentic sample, 153–154°. A portion, 0.544 g., was dissolved in 5 ml. of chloroform and examined in the polarimeter, α_{obsd} 0.00°. The infrared spectrum was identical with that of the compound obtained in the non-enzymatic hydrolysis.

(3) A solution of 0.795 g. (3.44 mmoles) of the ester IV, 0.104 g. of α-chymotrypsin and 1.5 ml. of buffer in 22 ml. of water was allowed to react at *p*H 7.8, 0.466 ml. of *N* NaOH being consumed in 210 min., 13.5% reaction, 0.821 ml. in 420 min., 23.9% reaction. The solution was examined in the polarimeter, α_{obsd} −0.49 ± 0.02°, α_{obsd} for α-chymotrypsin blank, −0.47 ± 0.02°.

Diethyl α-Benzyl-α-acetamidomalonate.—Sodium (1.2 g., 0.05 mole) was dissolved in 75 ml. of absolute ethanol, and 10.9 g. (0.05 mole) of diethyl acetamidomalonate was added, followed by 6.9 g. (0.055 mole) of benzyl chloride. The mixture was boiled for 4 hr., concentrated in vacuum to about 15 ml. and treated with 100 ml. of 0.5 *M* acetic acid. Crystals were collected, washed with cold water, dried and recrystallized from benzene–ethanol; 6.6 g. (0.021 mole), 43% yield, m.p. 105–105.5°, reported[17] 106°.

A suspension of 0.307 g. (1 mmole) of the ester in a solution of 2.0 ml. of 0.1 *M* Na_2HPO_4 and 0.300 g. of α-chymotrypsin in 20 ml. of water was brought to *p*H 7.8, stirred magnetically and observed in the *p*H stat. There was essentially no consumption of alkali.

(16) F. Zetzsche, H. E. Meyer, H. Overbeck and W. Nerger, *Ber.*, **71**, 1512 (1938).

(17) H. R. Snyder, J. F. Shekleton and C. D. Lewis, *J. Am. Chem. Soc.*, **67**, 310 (1945).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, BRANDEIS UNIVERSITY, WALTHAM 54, MASS.]

Requirements for Stereospecificity in Hydrolysis by α-Chymotrypsin. IV. The Hydroxyl Substituent. Absolute Configurations[1]

BY SAUL G. COHEN AND EZRA KHEDOURI

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dl-Ethyl lactate (I), diethyl α-hydroxymalonate (II), *dl*-ethyl β-hydroxybutyrate (III), *dl*-ethyl β-phenyl-β-hydroxypropionate (IV), dimethyl β-hydroxyglutarate (V) and diethyl β-hydroxyglutarate (VI) have been hydrolyzed by α-chymotrypsin. Compounds I, II and III were hydrolyzed with no stereospecificity, compound III hydrolyzing very slowly. Compounds IV, V and VI were hydrolyzed stereospecifically, leading to optically pure (−)-β-phenyl-β-hydroxypropionic acid and the (−)-monoalkyl hydrogen β-hydroxyglutarates, which were characterized as their 1,3-bis-(*p*-dimethylaminophenyl)-ureides. The stereospecific hydrolyses of V and VI and of the previously studied diethyl β-acetamidoglutarate proceed in the L-sense. The effectiveness of alpha and beta hydroxyl and acetamido groups in leading to stereospecific hydrolysis by α-chymotrypsin is compared.

Study of the hydrolysis of α-chymotrypsin of a number of esters containing α- and β-acetamido substituents—diethyl α-acetamidomalonate,[2] diethyl β-acetamidoglutarate,[3] ethyl α-acetamido-

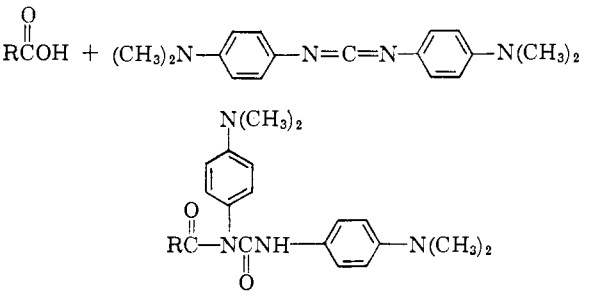
(1) We are pleased to acknowledge support of this work by the Division of Research Grants, National Institutes of Health, RG 4584.

(2) S. G. Cohen and L. H. Klee, *J. Am. Chem. Soc.*, **82**, 6038 (1960).

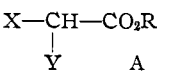
(3) S. G. Cohen and E. Khedouri, *ibid.*, **83**, 1093 (1961).

propionate,[4] ethyl β-acetamidobutyrate,[4] ethyl β-phenyl-β-acetamidopropionate[4] and diethyl acetamidomethylmalonate[4] has led to the following analysis: (1) The β-aryl and α-acylamido substituents of typical substrates for α-chymotrypsin[5] are important for high chemical reactivity but are not required for stereospecificity. (2) An α- or β-acetamido group at a center or developing center of asymmetry is sufficient to lead to stereospecificity when hydrolysis occurs. (3) In these acetamido compounds, the same factors lead to stereospecificity in hydrolysis of symmetric substrates of type Cabdd (the malonate and glutarate) as in the case of the common asymmetric substrates. (4) Specific interactions of a conformational diastereomeric character, of groups at the center or developing center of asymmetry in the substrate with groups at centers of asymmetry in the enzyme, may account for stereospecificity. In order to investigate further the structural requirements for stereospecificity, and the nature of the interactions between enzyme and substrate which lead to this specificity and thus, in part at least, to the hydrolytic action, it has seemed to us desirable to examine the effects of substituents other than the acylamido group on the stereospecificity and rates of hydrolysis of esters by α-chymotrypsin. We report at this time on stereospecificity in the hydrolysis of some α- and β-hydroxy esters. This affords comparison with the α- and β-acetamido esters reported previously.[2-4] In this work we have determined the absolute configuration in the asymmetric hydrolysis by α-chymotrypsin of the symmetric compound dimethyl β-hydroxyglutarate, and have obtained evidence as to the related asymmetric hydrolysis of diethyl β-acetamidoglutarate.[3]

We have examined the action of α-chymotrypsin on *dl*-ethyl lactate (I), diethyl α-hydroxymalonate (II), *dl*-ethyl β-hydroxybutyrate (III), *dl*-ethyl β-phenyl-β-hydroxypropionate (IV), dimethyl β-hydroxyglutarate (DMHG, V) and diethyl β-hydroxyglutarate (DEHG, VI). Compounds I and III were commercially available materials; compound II was prepared by esterification of tartronic acid; compound IV was prepared by the Reformatsky procedure from ethyl α-bromoacetate and benzaldehyde; DMHG (V) was prepared by reduction of dimethyl β-ketoglutarate with sodium borohydride; DEHG (VI) was prepared by hydrolysis of V to β-hydroxyglutaric acid, isolation of the acid and esterification to VI with ethanol. Hydrolysis of these esters by α-chymotrypsin was followed in a *p*H-stat—Radiometer Titrator and automatic buret—at *p*H 7.8 with magnetic stirring. The extent of non-enzymatic hydrolysis, usually small, was determined in each case as a correction factor. Optical rotations were observed in a Zeiss–Winkel polarimeter and were accurate to ±0.02°. A reagent which was useful in most cases for characterization of the acid hydrolysis products was 1,3-bis-(*p*-dimethylaminophenyl)-carbodiimide,[6] which converted the acids to the corresponding crystalline ureides



The α-Hydroxyl.—*dl*-Ethyl lactate (I) was hydrolyzed by α-chymotrypsin with no stereospecificity. In one run the hydrolysis was interrupted after 7.5 hours, 66% hydrolysis, and no optical rotation due to the reaction was found in the hydrolysis solution; the unhydrolyzed ester, which was recovered in high yield and characterized by its infrared spectrum, had no optical activity. A second similar run was allowed to proceed for 24 hours, 95% hydrolysis occurring, both enantiomorphs reacting with no evidence of two rate constants. A similar conclusion has been previously proposed.[7] Similarly, diethyl α-hydroxymalonate (II) was hydrolyzed with no stereospecificity, no change in the optical rotation of the hydrolysates being observed in several runs after 44, 81 and 92% hydrolysis of one ester group. In the latter run the hydrolysis product, ethyl hydrogen α-hydroxymalonate was isolated and found to have no optical activity. Although this compound might be expected to racemize rapidly, our experience with the corresponding α-acetamidomalonate[2] and α-acetoxymalonate[8] indicates that had it been formed asymmetrically it is very likely that we would have detected an optical rotation. Thus the α-hydroxyl substituent, unlike the α-acetamido group, does not lead to stereospecificity in hydrolysis of the substituted propionate and malonate esters. On the other hand, it has been reported that the α-hydroxyl and α-benzyl (or β-phenyl) groups do lead to some stereospecificity in the hydrolyses of D and L-enantiomorphs of esters of α-hydroxyhydrocinnamic acid, the L-enantiomorph being hydrolyzed more rapidly[9] than the D. This stereospecificity is not due solely to the α-benzyl group, since the corresponding D and L-α-chlorohydrocinnamate enantiomorphs are hydrolyzed by α-chymotrypsin at equal rates.[9] In α-substituted substrates of type A, if Y is acetamido, it has such



firm interaction with a functional group of the enzyme that asymmetric hydrolysis results, and X may be $-CH_3$, $-CO_2R$ or $-CH_2C_6H_5$. However, if Y is hydroxyl it interacts but feebly with the enzyme and asymmetric hydrolysis does not result unless the second substituent X is particularly effective either because of its size or because of some special interaction.

(4) S. G. Cohen, Y. Sprinzak and E. Khedouri, *J. Am. Chem. Soc.*, **83**, 4225 (1961).
(5) H. Neurath, G. Schwertand, *Chem. Revs.*, **46**, 69 (1950).
(6) F. Zetzche and W. Neiger, *Ber.*, **73B**, 467 (1940).
(7) I. Tinoco, *Arch. Biochem. and Biophys.*, **76**, 148 (1958).
(8) S. G. Cohen and E. Khedouri, unpublished results.
(9) (a) J. E. Snoke and H. Neurath, *Arch. Biochem.*, **21**, 351 (1949); (b) M. L. Bender and B. W. Turnquest, *J. Am. Chem. Soc.*, **77**, 4271 (1955).

The β-Hydroxyl.—*dl*-Ethyl β-hydroxybutyrate was hydrolyzed very slowly by α-chymotrypsin, only 25% hydrolysis being observed in one hydrolysis which was allowed to run for 40 hours. The hydrolysis showed no stereospecificity. The reaction solution itself showed no contribution to the optical rotation due to hydrolysis products. The unhydrolyzed ester was recovered (90%), identified by its infrared spectrum and found to have no optical activity. The acid produced, β-hydroxybutyric acid, was isolated in 26% yield, identified by its infrared spectrum which was identical with that of a synthesized authentic sample, and found to be optically inactive. It was further characterized as its 1,3-bis-(*p*-dimethylaminophenyl)-ureide which was optically inactive and identical with a synthesized sample.

However, the hydrolysis of other β-hydroxy esters was stereospecific. A suspension of *dl*-ethyl β-phenyl-β-hydroxypropionate was slowly hydrolyzed by α-chymotrypsin, the reaction slowing down very markedly and being interrupted when 93% of one enantiomorph had been hydrolyzed. The unhydrolyzed ester was recovered in high yield and found to be optically active, $[\alpha]^{22}D$ +40°, 2.8% in chloroform. The hydrolysis product (−)-β-phenyl-β-hydroxypropionic acid (VII) was recovered in almost quantitative yield and appeared to be optically pure, indicating high stereospecificity, $[\alpha]^{22}D$ −19.2°, 2.3% in ethanol, reported[10] −18.4°. This acid was treated with 1,3-bis-(*p*-dimethylaminophenyl)-carbodiimide and characterized as the substituted ureide, $[\alpha]^{22}D$ −15.8°, 1.9% in chloroform.

Dimethyl and diethyl β-hydroxyglutarates, compounds V and VI, were also hydrolyzed asymmetrically. Reactions were allowed to proceed to 92% and 85% hydrolysis of one ester group, at which point the rates had become very low, and the reaction solutions showed optical activity. The residual unhydrolyzed diesters were removed and the hydrolysis products methyl hydrogen β-hydroxyglutarate (VIII) and ethyl hydrogen β-hydroxyglutarate (IX) were isolated in 88 and 85% yields and found to be optically active, $[\alpha]^{22}D$ −1.7° and −1.8°, respectively. As will be shown below, these products appear to be optically pure and thus to have been formed with complete stereospecificity, the α-chymotrypsin distinguishing between the two "identical" ester groups in these β-hydroxyglutarates. The half-esters were each treated with 1,3-bis-(*p*-dimethylaminophenyl)-carbodiimide and characterized as the corresponding crystalline ureides, $[\alpha]^{22}D$ +4.2° and +4.4°, respectively, each formed in excellent yield.

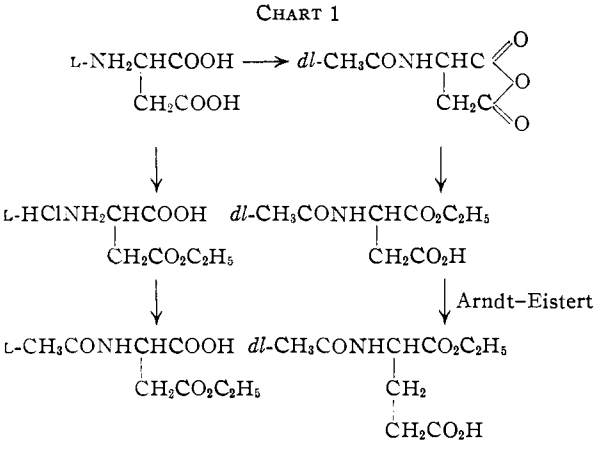
In β-substituted substrates of type B, the butyrates, with X = CH_3, led to no hydrolysis

$$\begin{array}{l} X\text{—}CHCH_2CO_2R \\ \quad\;\; | \\ \quad\;\; Y \quad B \end{array}$$

when Y was acetamido, and to slow non-stereospecific hydrolysis when Y was hydroxyl. It may be that a firm interaction of the β-acetamido group with the enzyme does not bring the ester group close to the nucleophilic center of the active site and reaction is not observed. A weaker interaction of the hydroxyl group allows slow non-stereospecific hydrolysis. However, in the β-substituted hydrocinnamate and glutarate derivatives, with X = C_6H_5 or RO_2CCH_2–, slow hydrolysis occurred when Y was either acetamido[3,4] or hydroxyl. The reactions were stereospecific in all four cases, and we note again[3] that no special mechanism is required to account for asymmetry in the reaction of the symmetric glutarates. The β-carbethoxymethyl group appears to be as effective sterically as β-phenyl and these larger groups facilitate the hydrolysis of the β-acetamido esters, possibly by causing distortion at the active site. On the other hand, the α-carbethoxyl group in the malonate, like the α-methyl in the propionate, does not lead to stereospecific hydrolysis of the respective α-hydroxyesters.

Absolute Configurations.—It is important to establish the absolute sense of the stereospecificity in the hydrolysis of these unusual substrates and in particular in the hydrolysis of the symmetrical esters of type Cabdd. Since the optically active product from hydrolysis of diethyl α-acetamidomalonate racemizes rapidly,[2] we have concentrated on the glutarates. (+)-Ethyl hydrogen β-acetamidoglutarate was obtained first,[3] and unsuccessful attempts were made to relate it to L-aspartic acid by the Arndt–Eistert reaction (Chart 1).

CHART 1



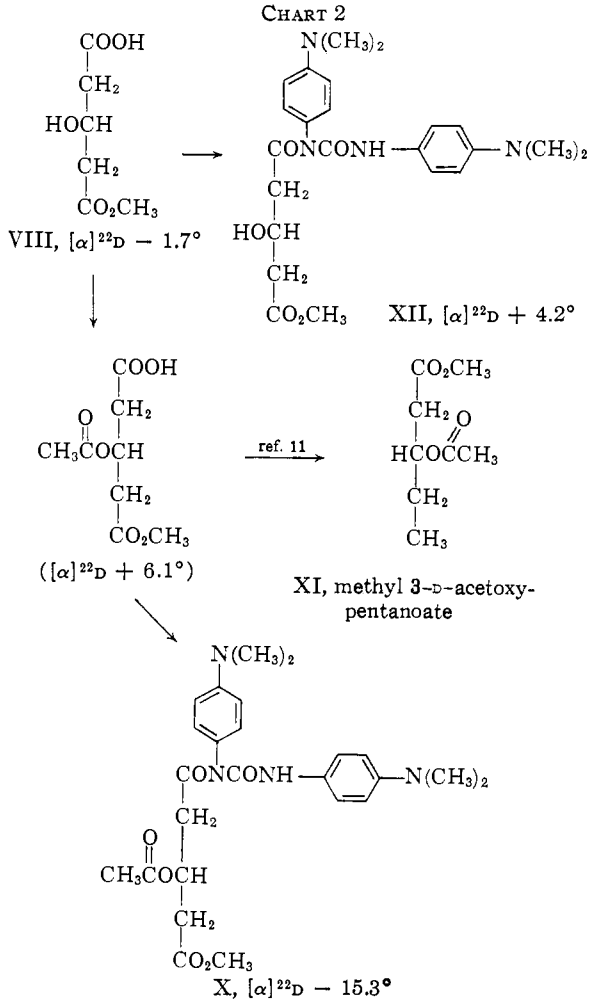
L-Aspartic acid was converted to N-acetylaspartic anhydride, racemizing in the process. This anhydride was treated with ethanol leading to a half ester, which was converted to the acid chloride and treated with diazomethane and silver oxide. This led apparently to α-ethyl-*dl*-N-acetylglutamic acid, since it had the correct analysis, but was not identical with active or *dl*-ethyl hydrogen β-acetamidoglutarate. The reaction of the acetylaspartic anhydride had apparently led to isolation of the α-ethyl monoester rather than to the desired β-ester. In a second sequence, L-aspartic acid was converted to the β-monoethyl ester hydrochloride and thence to the desired β-ethyl-L-N-acetylaspartic acid. However, attempts to carry out the Arndt–Eistert reaction on this failed, and this approach was abandoned.

We were able, however, to establish the absolute configuration of the (−)-methyl hydrogen β-

(10) H. Wieland, W. Koschara, E. Dane, J. Renz, W. Schwarze and W. Linde, *Ann.*, **540**, 103 (1939).

hydroxyglutarate (VIII). It was converted by acetylation to optically active methyl hydrogen β-acetoxyglutarate, and by treatment of this with 1,3-bis-(*p*-dimethylaminophenyl)-carbodiimide, to (−) - 1 - (3 - acetoxy - 4 - methoxycarbonylbutanoyl) - 1,3 - bis - (dimethylaminophenyl)-urea (X), $[\alpha]^{22}D$ −15.3° (Chart 2).

CHART 2



Compound X had been reported previously,[11] prepared by treatment of completely resolved (+)-methyl hydrogen β-acetoxyglutarate with the carbodiimide; $[\alpha]^{23}D$ −15.7 ± 0.4°. Comparison of the specific rotations indicates that the stereospecificity in the hydrolysis of dimethyl β-hydroxyglutarate by α-chymotrypsin had been essentially complete. This (+)-methyl hydrogen β-acetoxyglutarate had been converted by anodic coupling with acetic acid to the acetylated methyl ester of (−)-3-D-hydroxypentanoic acid (XI). Thus its absolute configuration had been established and it may be referred to as (+)-methyl 3-D-acetoxy-4-carboxybutanoate, or as (+)-3L-acetoxy-4-carbomethoxylbutanoic acid, Chart 2. In the latter nomenclature it is clear that the stereospecific hydrolysis by α-chymotrypsin of dimethyl β-hydroxyglutarate occurs in the L-sense.

This correlation was not repeated on (−)-ethyl hydrogen β-hydroxyglutarate, product of the α-chymotrypsin-catalyzed hydrolysis of diethyl β-hydroxyglutarate, the related ethyl hydrogen β-acetoxyglutarate not having been studied.[11] However, comparison of the specific rotations of (−)-methyl hydrogen β-hydroxyglutarate and its 1,3-bis-(*p*-dimethyl-aminophenyl)-ureide (XII), −1.7° and +4.2°, with the corresponding values of the ethyl compounds, −1.8° and +4.4°, indicates that the stereospecificity is high and in the same sense in the latter hydrolysis also.

Finally, comparison of the values of specific rotations of (+)-ethyl hydrogen β-acetamidoglutarate, formed by hydrolysis of diethyl β-acetamidoglutarate by α-chymotrypsin,[3] and its 1,3-bis-(*p*-dimethylaminophenyl)-ureide, +5.9° and −15.2°, respectively, with those of (+)-methyl hydrogen β-acetoxyglutarate and its corresponding ureide,[11] +6.1° and −15.7°, respectively, indicated that the stereospecific hydrolysis of the acetamidoglutarate also proceeded in the L-sense.

Experimental[12]

1,3-Bis-(*p*-dimethylaminophenyl)-carbodiimide.—N,N-Dimethyl-*p*-phenylenediamine, (80 g., 0.58 mole, Eastman Kodak Co.) was dissolved in 200 ml. of benzene, a solution of 126 g. (1.66 moles) of carbon disulfide in 50 ml. of benzene was added and the mixture was boiled under reflux for 16 hr. The solid was filtered and washed with benzene; N,N'-bis-(*p*-dimethylaminophenyl)-thiourea, 96 g., quantitative yield. This was triturated twice for 12 hours with 800-ml. portions of acetone, leading to colorless crystals, m.p. 186°, reported[6] 186–188°. A portion of this (20 g., 0.062 mole) was boiled for 20 minutes in 800 ml. of acetone with 0.40 g. of flowers of sulfur, treated with 30 g. (0.134 mole) of finely divided lead oxide under reflux for 15 minutes and stirred at room temperature for 2 hr. The mixture was filtered, the filtrate was concentrated to 75 ml. in vacuum, 60 ml. of 20–40° petroleum ether was added, and the mixture was cooled in ice and the unchanged thiourea was filtered off. The filtrate was concentrated in vacuum, leading to the carbodiimide which was crystallized from 100 ml. of 60–110° petroleum ether; 7.0 g. (0.025 mole), 40% yield, m.p. 88–89°, reported[6] 88–90°.

***dl*-Ethyl lactate** (Olin-Mathieson) was distilled, b.p. 155°. Ethyl lactate (1.025 g., 8.68 mmoles), 2.5 ml. of 0.1 *M* Na_2HPO_4, 0.300 g. of α-chymotrypsin and 20 ml. of water were brought to *p*H 7.8, and the hydrolysis was followed in the *p*H stat at room temperature (29°). In 7.5 hr., 5.68 ml. of *N* NaOH was consumed (corrected for 1% hydroxide ion catalyzed hydrolysis), 66% hydrolysis. The optical rotation of the solution was identical with that of a chymotrypsin blank. The solution was saturated with sodium sulfate and extracted with ether, leading to 0.22 g. (1.85 mmoles), 63% recovery, of ethyl lactate, which had no optical activity; infrared spectrum in chloroform identical with that of an authentic sample with peaks at 2.82(w), 3.35(m), 5.77(s), 6.87(w), 7.25(w), 7.92(m), 8.85(m), 9.60(m), 9.81(mμ). In another run, starting with the same quantities of starting materials, hydrolysis was allowed to proceed for 24 hr., 8.22 ml. of *N* NaOH was consumed, 94.7% hydrolysis.

Diethyl α-Hydroxymalonate.—A solution of tartronic acid (30 g., 0.25 mole, Nutritional Bichemicals Corp.), and 2 ml. of concentrated sulfuric acid in 700 ml. of ethanol was boiled under reflux for 18 hr., concentrated to 50 ml., neutralized with sodium bicarbonate, dried over sodium sulfate and extracted with ether. The extracts were dried, concentrated in vacuum and distilled; diethyl α-hydroxymalonate (35 g., 0.20 mole), 85% yield, b.p. 120–122° (15 mm.), reported[13] 120.5–121° (15 mm.).

A solution of 1.027 g. (5.83 mmoles) of the ester, 2.5 ml. of 0.1 *M* Na_2HPO_4 and 0.200 g. of α-chymotrypsin in 20 ml. of water was followed at *p*H 7.8 at 27° in the *p*H stat. In 2 hr., 5.4 ml. of *N* NaOH was consumed (corrected for 2% hydroxide ion catalyzed hydrolysis), 92% hydrolysis of one ester

(11) K. Serck-Hanssen, *Arkiv. Kemi*, **10**, 135 (1956).

(12) Melting points are uncorrected. Elementary analyses are by Dr. S. M. Nagy, Massachusetts Institute of Technology.

(13) A. Pinner, *Ber.*, **18**, 752 (1885).

group. The optical rotation of the solution was identical with that of a chymotrypsin blank. The solution was extracted with ether, brought to *p*H 2 with *N* HCl and taken to dryness in vacuum. The residue was extracted with ether, the extracts were concentrated leading to an oil residue, apparently ethyl hydrogen α-hydroxymalonate (0.84 g., 5.7 mmoles), 97% yield, α_{obsd}, 0.00°, 10% in chloroform. Repeated attempts to prepare a solid derivative of this by treatment with 1,3-bis-(*p*-dimethylaminophenyl)-carbodiimide failed. The infrared spectrum was obtained in chloroform: 2.90(w), 3.12(w), 3.47(m), 5.75(s), 6.25(w), 6.82(w), 6.92(w), 7.30(m), 8.50(m), 9.35(m), 11.70(m)μ.

dl-Ethyl β-hydroxybutyrate (Eastman Kodak Co.) was redistilled, b.p. 179°. It was found to be inert at *p*H 7.8 in water. A solution of 1.070 g. (8.1 mmoles) of the ester, 1.5 ml. of 0.1 *M* Na_2HPO_4 and 0.300 g. of α-chymotrypsin was made up to 20 ml. with distilled water, brought to *p*H 7.8 and followed in the *p*H state, 1.46 ml. of *N* NaOH being consumed in 24 hr., 18% hydrolysis; 2.02 ml., 25% hydrolysis in 40 hr. The optical rotation of the solution was compared to that of an α-chymotrypsin blank, showing no rotation due to products. The solution was saturated with sodium sulfate and extracted with ether; the extracts were dried and concentrated leading to 0.675 g. (5.1 mmoles), 90% recovery, of *dl*-ethyl β-hydroxybutyrate, α_{obsd} 0.00°, in chloroform, acetone and water. The infrared absorption spectrum in chloroform was identical with that of the starting material, showing bands at 2.88(m), 3.40(m), 5.83(s), 6.96(m), 7.12-(m), 7.38(m), 7.55(m), 7.88(m), 8.48(s), 9.0(m), 9.50(m), 9.75(m)μ.

The reaction solution, which had been extracted, was brought to *p*H 2 with *N* HCl and extracted with ten 50-ml. portions of ether, which were combined, dried and concentrated, leading to 0.050 g. (0.48 mmole), 26% recovery, of an oil, *dl*-β-hydroxybutyric acid, α_{obsd} 0.00° in chloroform, water and acetone; its infrared spectrum was identical with that of synthetic *dl*-β-hydroxybutyric acid.

The acid recovered from another run (0.050 g., 0.48 mmole) in 5 ml. of anhydrous ether was treated with 0.135 g. (0.48 mmole) of 1,3-bis-(*p*-dimethylaminophenyl)-carbodiimide in 10 ml. of ether under reflux for 3 hr. The solution was cooled in ice, leading to 1-(*dl*-3-hydroxybutanoyl)-1,3-bis-(*p*-dimethylaminophenyl)-urea (0.145 g., 0.38 mmole), 78% yield, m.p. 146–147°, α_{obsd} 0.00° in chloroform.

Anal. Calcd. for $C_{21}H_{28}N_4O_3$: C, 65.60; H, 7.34; N, 14.57. Found: C, 65.51; H, 7.40; N, 14.66.

dl-β-Hydroxybutyric acid was prepared by treatment of 5.0 g. (0.038 mole) of ethyl *dl*-β-hydroxybutyrate with 1.6 g. (0.04 mole) of sodium hydroxide in 15 ml. of methanol. The mixture was distilled to dryness in 0.5 hr. on the water-bath. The residue was dissolved in 5 ml. of water, brought to *p*H 1 with concentrated HCl, saturated with salt and extracted with ether. The extract was dried and concentrated, leading to *dl*-β-hydroxybutyric acid (3.0 g., 0.029 mole), 77% yield. Its infrared absorption spectrum in chloroform showed bands at 2.85(w), 3.15(w), 3.40(w), 3.50(w), 5.85(s), 6.07(w), 6.20(m), 6.30(m), 6.60(s), 6.93(w), 7.40(m), 8.45-(m), 8.65(m), 9.50(w), 10.60(m)μ. It was converted to the ureide with 1,3-bis-(*p*-dimethylaminophenyl) carbodiimide, m.p. and mixed m.p. with that from the product of enzymatic hydrolysis 146–147°.

dl-Ethyl β-phenyl-β-hydroxypropionate was prepared from 18.7 g. (0.29 mole) of zinc, 39.0 g. (0.23 mole) of ethyl bromoacetate and 30.2 g. (0.29 mole) of benzaldehyde, according to directions in the literature[14]; b.p. 134–136° (2 mm.), reported 151–154° (11–12 mm.), 32.3 g. (0.17 mole), 71% yield.[15]

A suspension of 1.031 g. (5.31 mmoles) of this ester in a solution of 0.200 g. of α-chymotrypsin and 1 ml. of 0.1 *M* Na_2HPO_4 in 19 ml. of water was allowed to react in the *p*H stat, at *p*H 7.8, 28°, 2.48 ml. of *N* NaOH being consumed in 15 hours, at which time the reaction was proceeding at less than one-tenth of the initial rate. The suspension was extracted with ether, the extracts were dried and concentrated leading to optically active starting ester (0.47 g., 2.42 mmoles), 85% yield. The infrared spectrum in chloroform was identical with that of the starting material: 2.85-(w), 3.38(w), 5.80(s), 6.69(w), 6.88(w), 7.13(m), 7.28(m), 7.57(m), 8.40(m), 9.45(w), 9.75(m)μ. The optical rotation was observed, 0.114 g. in 4 ml. of chloroform, α_{obsd} +1.98°, $[\alpha]^{22}D$ +34.8°; corrected for 93% hydrolysis of one enantiomorph, $[\alpha]^{22}D$ +40°, reported[16] $[\alpha]D$ +19.17° (pure compound).

The aqueous solution was brought to *p*H 2 and taken to dryness in vacuum, and the residue was extracted with acetone, leading to (−)-β-phenyl-β-hydroxypropionic acid (0.40 g., 2.41 mmoles), 97% yield, m.p. 117–118° from chloroform, α_{obsd} −1.39°, 0.111 g. in 4 ml. of ethyl acetate, $[\alpha]^{22}D$ −25.1; α_{obsd} −0.89°, 0.116 g. in 5 ml. of ethanol, $[\alpha]^{22}D$ −19.2°; reported[10] m.p. 116°, $[\alpha]D$ −18.4°, 2.4% in alcohol.

Anal. Calcd. for $C_9H_{10}O_3$: C, 65.05; H, 6.07. Found: C, 65.05; H, 6.12.

A solution of 0.166 g. (1.00 mmole) of this acid in 10 ml. of acetone was treated with 0.28 g. (1.00 mmole) of 1,3-bis-(*p*-dimethylaminophenyl)-carbodiimide in 10 ml. of acetone under reflux for 3 hr. The precipitate was collected; 0.262 g. (0.59 mmole), 59% yield, m.p. 177–178° dec., from acetone; α_{obsd} −0.60°, 0.076 g. in 4 ml. of chloroform, $[\alpha]^{22}D$ −15.8°.

Anal. Calcd. for $C_{26}H_{30}N_4O_3$: C, 69.93; H, 6.77. Found: C, 69.47; H, 6.53.

Dimethyl β-Hydroxyglutarate (DMHG).—A solution of 50 g. (0.29 mole) of dimethyl β-ketoglutarate (Eastman Kodak Co.) in 50 ml. of methanol was treated with a solution of 5 g. (0.13 mole) of sodium borohydride in 50 ml. of water and 1 drop of 40% sodium hydroxide, over a period of 45 min. at 25°. Excess borohydride was carefully destroyed with 5 *N* sulfuric acid, the solution was diluted to 350 ml. with water, brought to *p*H 7 with dilute NaOH and concentrated in vacuum to 100 ml. This was extracted with ether, the extract was washed with water, dried and distilled leading to DMHG (45 g., 0.26 mole), 95% yield, b.p. 138–140° (8 mm.), reported[17] 138–139° (8 mm.).

A solution of 1.004 g. (5.67 mmoles) of DMHG, 2.0 ml. of 0.1 *M* Na_2HPO_4 and 0.500 g. of α-chymotrypsin (Worthington, salt free) in 20 ml. of distilled water was followed in the *p*H stat at *p*H 7.8, 4.33 ml. of *N* NaOH being consumed in 5 hr., 76% hydrolysis, 5.24 ml. in 15 hr., 92% hydrolysis of 1 ester group. The optical rotation was compared with that of a solution of α-chymotrypsin treated in the same way, showing a net contribution due to the product of hydrolysis, α_{obsd} −0.92°.

The solution was extracted with ether to remove unchanged DMHG, brought to *p*H 2 with *N* HCl, and taken to dryness in vacuum at room temperature. The residue was extracted with ether, and from the extract was obtained an oil, (−)-methyl hydrogen β-hydroxyglutarate (0.75 g., 4.62 mmoles), 88% yield (cor.), α_{obsd} −0.42°, 12.5% in chloroform, $[\alpha]^{22}D$ −1.7°. A portion of this product (0.300 g., 1.85 mmoles) in 20 ml. of anhydrous ether was boiled under reflux for 3 hr. with a solution of 0.520 g. (1.85 mmoles) of 1,3-bis-(*p*-dimethylaminophenyl)-carbodiimide in 25 ml. of ether. The precipitate was collected, washed with cold ether and crystallized from warm ether, leading to (+)-1-(3-hydroxy-4-methoxy-carbonylbutanoyl)-1,3-bis-(*p*-dimethylaminophenyl)-urea (0.64 g., 1.44 mmoles), 78% yield, m.p. 127–128°, α_{obsd} +0.23°, 2.7% in chloroform, $[\alpha]^{22}D$ +4.2°. The infrared spectrum in chloroform showed peaks at 2.78(w), 2.90(w), 3.12(w), 3.40(m), 5.85(s), 6.08(w), 6.22(m), 6.60(s), 6.95(m), 7.40(m), 8.38(m), 8.65(m), 10.60(w)μ.

Anal. Calcd. for $C_{23}H_{30}O_5N_4$: C, 62.42; H, 6.83; N, 12.66. Found: C, 62.41; H, 6.96; N, 12.54.

Diethyl β-Hydroxyglutarate (DEHG).—Dimethyl β-hydroxyglutarate (75 g., 0.43 mole) and 56 g. (1 mole) of KOH were dissolved in 180 ml. of methanol and the solution was concentrated to dryness in the steam-bath in 30 minutes. The solid was dissolved in 100 ml. of water, brought to *p*H 1.5 with concentrated HCl, taken to dryness in vacuum and extracted with ethyl acetate. The extract was dried, concentrated and triturated with a little ether, leading to β-hydroxyglutaric acid (50 g., 0.34 mole), 80% yield, m.p. 94–95°, reported[18] 95°. The acid was boiled for 24 hr. in

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(15) This compound was prepared by Dr. Y. Sprinzak.

(16) J. Kenyon, H. Phillips and G. R. Shutt, *J. Chem. Soc.*, 1663 (1935).

(17) R. P. Evstigneeva, R. S. Livshitz, M. S. Bainova, L. I. Zakharbin and N. A. Preobrashenskii, *Zhur. Obshch. Khim.*, **22**, 1467 (1952); *C. A.*, **47**, 5949 (1953).

(18) H. V. Pechmann and K. Jenish, *Ber.*, **24**, 3250 (1891).

excess absolute ethanol and 2 ml. of sulfuric acid, concentrated to 100 ml., neutralized with sodium bicarbonate, extracted with ether, dried and distilled leading to diethyl β-hydroxyglutarate (38 g., 0.19 mole), 55% yield, b.p. 138–141° (10 mm.), reported[11] 138–142° (10 mm.).

DEHG (1.096 g., 5.39 mmoles) was hydrolyzed by α-chymotrypsin as described above for DMHG, 55% hydrolysis being observed after 5.5 hr., 85% hydrolysis of 1 ester group after 15 hr. The optical rotation in the solution due to the product was −0.73°. The solution was worked up in the same way, leading to (−)-ethyl hydrogen β-hydroxyglutarate (0.675, 3.82 mmoles), 85% yield (cor.), α_{obsd} −0.41°, 11.5% in acetone, $[\alpha]^{22}D$ −1.8°. A portion of this (0.250 g., 1.42 mmoles) in 15 ml. of ether was treated with a solution of 0.400 g. (1.43 mmoles) of 1,3-bis-(*p*-dimethylaminophenyl)-carbodiimide in 15 ml. of ether under reflux for 3 hr. The solution was taken to dryness and the residue crystallized from 60–110° petroleum ether; (+)-1-(3-hydroxy-4-ethoxycarbonylbutanoyl) - 1,3 - bis - (*p* - dimethylaminophenyl) - urea (0.520 g., 1.14 mmoles), 80% yield, m.p. 97–98°, α_{obsd} +0.22°, 2.5% in chloroform, $[\alpha]^{22}D$ +4.4°.

Anal. Calcd. for $C_{24}H_{32}O_5N_4$: C, 63.14; H, 7.07; N, 12.27. Found: C, 63.23; H, 7.07; N, 12.20.

Attempted Arndt–Eistert Synthesis of (+)-Ethyl Hydrogen β-Acetamidoglutarate.—(a) Acetic anhydride (25 ml.) was added to a solution of L-aspartic acid (5 g., 0.038 mole) in 100 ml. of hot water, the mixture was cooled, 75 ml. of acetic anhydride was added, the mixture was stirred at 20° for 6 hours, and concentrated, leading to 6.7 g. of a gum. This was heated with 40 ml. of acetic anhydride at 95–100° for 20 minutes, filtered, concentrated to half its volume, and cooled, leading to optically inactive N-acetylaspartic anhydride, 2.75 g., 32% yield, m.p. 143–146°, reported[19] 143–145° (reported[19] for the L-anhydride, 170–173°).

The anhydride above (2.5 g., 0.016 mole) was boiled in ethanol for 6 hours and concentrated, and crystallized several times from acetone–ether–petroleum ether, leading to α-ethyl-*dl*-N-acetylaspartic acid (1.75 g., 0.0086 mole), 54% yield, m.p. 102–103°.

Anal. Calcd. for $C_8H_{13}NO_5$: C, 47.3; H, 6.4; N, 6.9. Found: C, 47.6; H, 6.4; N, 6.7.

In addition a considerable quantity of lower melting material and oil was found, possibly the desired β-ester.

An Arndt–Eistert reaction was carried out on the α-ethyl-*dl*-N-acetylaspartic acid according to a standard procedure.[20] Purified thionyl chloride, 5 ml., was added to a solution of 0.50 g. (0.0024 mole) of the aspartic half ester in 5 ml. of chloroform, the solution was boiled for 0.75 hour and concentrated in vacuum. The residue was dissolved in 20 ml. of ether, added at 0–5° to a solution of 0.145 g. of diazomethane in 50 ml. of ether, and allowed to stand overnight. The solvents were evaporated, the residue was dissolved in 10 ml. of dioxane and added slowly at 50–60° to 15 ml. of water containing 0.15 g. of Ag_2O, 0.30 g. of Na_2CO_3 and 0.30 g. of $Na_2S_2O_3 \cdot 5H_2O$. The mixture was heated to 95°, cooled, acidified with dilute nitric acid, evaporated to dryness, taken up in water and extracted with ether and with ethyl acetate. The latter led to an oil, 0.4 g., which was decolorized in acetone and crystallized from acetone–petroleum ether, leading to α-ethyl-*dl*-N-acetylglutamic acid (0.063 g., 2.7 mmoles), 12% yield, m.p. 98°.

Anal. Calcd. for $C_9H_{15}NO_5$: C, 49.7; H, 6.9. Found: C, 49.3; H, 6.8.

(b) L-Aspartic acid (5 g., 0.038 mole) was suspended in 50 ml. of absolute ethanol and 8 g. of anhydrous hydrogen chloride was added, solution resulting. This was cooled and concentrated in vacuum, leading to β-ethyl-L-aspartic acid hydrochloride (2.8 g., 0.014 mole), 37% yield, m.p. 196–199°, reported[21] 198–200°.

This product was dissolved in 50 ml. of water, neutralized with solid sodium acetate, and treated with 50 ml. of acetic anhydride, as described above, leading to β-ethyl-L-N-acetylaspartic acid (1.5 g., 0.0074 mole), 52% yield, m.p. 109–110°, from acetone–ether, α_{obsd} +0.98°, 5.84% in ethanol, $[\alpha]^{22}D$ +8.4°.

Anal. Found: C, 47.3; H, 6.1; N, 6.8.

Attempts were made to carry out the Arndt–Eistert reaction on this using (a) thionyl chloride and (b) oxyalyl chloride in the first step, followed by the usual procedure.

Absolute Configuration of β-Substituted Glutarate Half Esters.—(1) (−)-Methyl hydrogen β-hydroxyglutarate (0.300 g., 1.85 mmoles) was dissolved in 5 ml. of pyridine, cooled to 0°, and treated with a solution of 0.65 g. of acetic anhydride in 5 ml. of pyridine at 0° for 30 min. The solution was concentrated in vacuum, treated with 5 ml. of water, brought to *p*H 2 with *N* HCl, and concentrated in vacuum. The residue was extracted with dry ether and the extract was concentrated, leading to an oil residue, methyl hydrogen β-acetoxyglutarate (0.225 g., 1.11 mmoles), 60% yield. To 0.200 g. (1.00 mmole) of this in 15 ml. of ether was added 0.280 g. (1.00 mmole) of 1,3-bis-(*p*-dimethylaminophenyl)-carbodiimide in 10 ml. of ether, and the solution was boiled for 3 hr. The solution was filtered and the filtrate was cooled in ice. Crystals were collected and washed with ether, (−)-1-(3-L-acetoxy-4-methoxycarbonylbutanoyl)-1,3-bis-(*p*-dimethylaminophenyl)-urea, m.p. 142°, α_{obsd} −0.90°, 2.95% in chloroform, $[\alpha]^{22}D$ −15.3°, reported[11] m.p. 142°, $[\alpha]D$ −15.7 ± 0.4, 5% in chloroform. The infrared spectrum in chloroform showed the absorption bands at 3.15(w), 3.55(m), 5.82(s), 5.86(s), 6.22(m), 6.65(s), 6.98(m), 7.40(m), 8.15(w), 8.70(m), 9.70(m), 10.60(m) μ.

(2) A solution of 0.100 g. (0.46 mmole) of (+)-ethyl hydrogen β-acetamidoglutarate[3] in 15 ml. of acetone was treated with a solution of 0.130 g. (0.46 mmole) of 1,3-bis-(*p*-dimethylaminophenyl)-carbodiimide in 15 ml. of ether under reflux for 3 hr. The solvent was evaporated and the residue was crystallized from ether and a little chloroform, leading to (−)-1-(1-3-acetamido-4-ethoxycarbonylbutanoyl)-1,3-bis-(*p*-dimethylaminophenyl)-urea (0.185 g., 0.37 mmole), 80.5% yield, m.p. 118–119°, α_{obsd} −0.89° 2.95% in chloroform, $[\alpha]^{22}D$ −15.2°.

Anal. Calcd. for $C_{26}H_{35}N_5O_5$: C, 62.76; H, 7.09; N, 14.08. Found: C, 62.44; H, 7.28; N, 13.98.

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(21) T. Curtius and F. Koch, *J. prakt. Chem.*, [2] **38**, 473 (1888).